Background and Purpose - The purpose of this study was to analyze whether different therapeutic approaches (drug cocktail vs. single-agent treatments) would provide similar neuroprotection in two different models of ischemic injury.

Methods- Male C57BL/6 mice were subjected to transient or permanent middle cerebral artery occlusion. The size of ischemic lesion and the glial responses were analyzed. In our treatments we used three drugs (alone or in combination) aiming distinct pharmacological targets; minocycline - an antibiotic with anti-inflammatory properties, riluzole – a glutamate antagonist, and nimodipine, a blocker of voltage-gated calcium channels.

Results- The drug cocktail approach conferred significantly more efficient neuroprotection than any of the cocktail components tested alone. Administered 2 hr after transient or permanent MCAO, the three-drug cocktail significantly decreased the size of infarction (by~ 65% after transient and by ~35% after permanent ischemia). In addition, some other interesting observations emerged from this study. First, treatment with the glutamate antagonist riluzole alone was beneficial in transient, but not in the model of permanent ischemia. Second, immunohisthological analysis of the brain sections 72 hr after stroke revealed a marked difference in the inflammatory - and tissue responses to therapies between transient and permanent ischemia.

Conclusions- Our results suggest that simultaneous targeting of several pathophysiological pathways involved in the evolution of ischemic injury may represent a rational therapeutic approach for stroke. In addition, our results also revealed that different tissue- and therapy- responses may be associated with the evolution of ischemic injury after transient and permanent ischemia.

Key words: minocycline, drug cocktail, mice, stroke, microglia/macrophages, caspase-3

Introduction

At the present, although important progress has been made in understanding the molecular pathway that lead to ischemic cell death, the current clinical treatments remain poorly effective. The trombolysis using recombinant tissue plasminogen activator (tPA) remains the only therapy for acute stroke approved by FDA (Dirnagl et al., 1998; STAIR, 1999; Lo et al., 2003). According to a current view, treatment of stroke is suboptimal without combining neuroprotection with clot-lysing therapy: the quest for effective neuroprotective treatments therefore remains an urgent priority (Grotta, 2001a; Grotta, 2001b; Gladstone et al., 2002; Lo et al., 2003).

Brain damage following transient or permanent ischemia results from a series of pathophysiological events that evolve in time and space. Bearing in mind that several pathways leading to a neuronal death are activated by cerebral ischemia, a combination of drugs rather than single-drug treatment may be required for efficient neuroprotection (Dirnagl et al.,1998; Choi, 2000; Ginsberg, 2003; Lo et al., 2003). Therefore, we designed a drug cocktail that simultaneously acts on distinct pharmacological targets during the evolution of ischemic injury. This drug cocktail consists of minocycline - an antimicrobial agent with anti-inflammatory properties, riluzole – a glutamate antagonist, and nimodipine, a voltage-gated calcium channel blocker. We recently demonstrated that such a pharmacological approach was remarkably effective in a mouse model of amyotrophic lateral sclerosis (Kriz et al., 2003). Furthermore, at present the differences in brain responses to ischemic injury between transient and permanent ischemia remain incompletely characterized and the therapeutic significance of these pathophysiological differences is unclear. Therefore, we investigated the efficacy of our treatment in two different experimental paradigms: reperfusion

injury that develops after transient middle cerebral artery occlusion (MCAO) and ischemic injury associated with permanent MCAO.

Here we report that the drug cocktail approach conferred significantly more efficient neuroprotection than any of the cocktail components tested alone. In addition our findings suggest that the pathophysiology of ischemic lesions and the mechanisms of neuroprotection may differ in transient and permanent cerebral ischemia.

Material and methods

Experimental animals

All experiments were carried out on adult (2-3 months old) male C57BL/6 mice (Charles River St-Constant, QC). All experimental procedures were according to the guidelines of the Canadian Council for Animal Care.

Surgical procedures

Unilateral transient focal cerebral ischemia was induced by intraluminal filament occlusion of the left middle cerebral artery (MCAO) during 1 hour. The MCAO was carried out in male C57Bl/6 mice (20-25 g) as previously described (Belayev et al., 1999; Baeulieu et al., 2001). The animals were anesthetized with ketamine/xylazine 100/20 mg/kg i.p.. To avoid cooling, the body temperature was regularly checked and maintained at 37°C with an infrared heating lamp and a heating pad. The left common carotid artery and ipsilateral external carotid artery (ECA) were exposed through a midline neck incision and were carefully isolated from surrounding tissue. The ECA was dissected farther distally and coagulated. The internal carotid artery (ICA) was isolated

and carefully separated from the adjacent tissue. 6-0 silk suture was tied loosely around the immobilized ECA stump and a 12 mm length of 6-0 silicon-coated monofilament suture was inserted via the proximal ECA into the ICA and then into the circle of Willis, thus occluding the MCA. One hour after occlusion, the intraluminal suture was carefully removed. The same surgical procedures were followed for the permanent MCAO, with the exception that 6-0 silicon coated monofilament was left in the ICA. The neck incision was closed with silk sutures and the mice were allowed to survive for 24-72 hours or 7 days after surgery.

Treatment protocol

Minocycline 50 mg/kg, riluzole 1 mg/kg and nimodipine 0.5 mg/kg alone or as a three-drug cocktail were administered (i.p.) 2 hr after MCA occlusion whereas control (non-treated) mice were injected with saline. The animals were injected once per day, for a maximum of 3 days: The doses were comparable (in some cases lower) to the doses previously described in literature. All three compounds were purchased from Sigma (Oakville, ON, Canada).

Size of infarct

The size of the infarct was estimated in at least 6 mice from each experimental group. The mice were sacrificed by overdose of anesthetic, the brains were quickly removed, chilled at -80 °C for few minutes and placed in a mouse brain mold (Stoelting). The brains were cut in 1 mm coronal sections, immersed in a 2% solution of 2,3,5 tryphenyltetrazolium chloride (TTC) (Sigma, Oakville, ON) dissolved in saline and stained for 20 min at 37° C in the dark. The relative size of the infarction was measured by using the Scion Image -processing and analysis program (Scion Corp. Frederick, MD), calculated in arbitrary units (pixels) and expressed as a percentage of the control, non-stroked area in the contralateral non-ischemic hemisphere. The total size of infarction

was obtain by numeric integration of area of marked pallor measured in six consecutive 1-mm coronal sections affected by MCA occlusion, with appropriate correction for brain edema.

Immunocytochemistry

Mice were killed by overdose of anesthetic, perfused with 16mg/L sodium cacodylate buffer (pH 7.4) followed by fixative (3% glutaraldehyde) as previously described (Kriz et al., 2002; Kriz et al., 2003). Tissues were incubated overnight at room temperature with the following primary antibodies: anti-glial fibrillary acidic protein monoclonal antibody (anti-GFAP 1:200 dilution; Sigma, Oakville, Canada), anti-mouse Mac2 rat monoclonal antibody (TIB-166) distributed by ATcc (1:500 dilution; Manassas, VA), and anti-cleaved caspase3 rabbit polyclonal antibody (1:500 dilution, Cell Signaling Tech., New England Biolab) – all in phosphate-buffered saline/bovine serum albumin. The labeling was developed using vector ABS kit (Vector Laboratories, Burlington,. ON, Canada) and Sigma-Fast tablets (Sigma, Oakville, ON, Canada).

Monitoring

To examine clinical recovery of mice neurological deficits were investigated, by a blinded investigator, using a battery of tests of sensorimotor deficits (Rogers et al., 1997). Each animal was given a functional score that reflected the extent of neurological deficit. The animals were monitored during the period of postoperative recovery starting at 1, 3 or 7 days after the stroke.

Statistical analysis

All data are expressed as mean ± standard error (SEM). Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by post hoc comparison (Bonferroni) test ($p \leq 0.05$).

Results

The three-drug cocktail is more effective than treatments with minocycline, riluzole or nimodipine

To compare the neuroprotective effects of the three-drug cocktail and single-agent treatments, we first measured the size of infarction following different therapeutic protocols (see Methods). All our treatments were initiated 2 hours after stroke. As shown in Fig. 2A and 2B, the three-drug treatment significantly decreased the size of infarction 24 hours after transient MCAO, 22.5±1.8% *vs* 46.7±1.9% in untreated animals (n=7-8, p=1.6^{-6}), and after permanent ischemia, 35.2±4.2% *vs* 77.3±4.0%, respectively (n=6, p= 0.003). As further revealed in our single drug treatments, 24 hours after transient MCAO, minocycline significantly decreased the size of infarction by ~ 25 %, 35.6±1.03% *vs* 46.7± 1.96% (n=5-8 and p=0.01). Treatment with low doses of riluzole had a similar effect (infarct area was 32.4±3.4%, n=5-8, p=0.007) whereas treatment with nimodipine alone did not have an effect (Figure 2A). Importantly, the three-drug cocktail was significantly more efficient in decreasing the size of the ischemic injury than any of the single drug treatments (Figure 2A). The same therapeutic protocols were followed in the mouse model of permanent MCAO. As demonstrated in Fig. 2B, treatment with minocycline significantly decreased (by ~ 35%) the size of ischemic injury 24 hr after permanent MCAO, 49.8±2.6% *vs* 77.3±4.0 %, control (n=6, p=4.6^{-4}). To our surprise, treatment with riluzole had no effect on the size of the ischemic lesion in the model of permanent ischemia. Interestingly, the treatment with the three-drug cocktail was again significantly

more effective in decreasing the size of ischemic lesion after permanent ischemia than treatments with minocycline, riluzole or nimodipine alone (Fig. 1B).

Three-drug cocktail provides long-term neuroprotection against cerebral ischemia and improves clinical recovery

To study the effects of the three-drug treatment on the maturation of the ischemic lesion, we measured the size of the ischemic lesions 72 hr after transient and permanent MCAO and 7 days following transient MCAO (unfortunately the conditions of non-treated mice subjected to permanent MCAO started to deteriorate rapidly after day 4; therefore they were excluded from the experiments). As shown in the Fig. 3A and B, 72 hours after transient MCAO treatment with the cocktail reduced the size of infarction by ~65 % as compared to non-treated control mice, 19.4± 2.1 *vs* 51.4±2.8%, respectively (n=5, p=2.2^{-4}) and by ~ 30% after permanent MCAO, 52.9± 4.5% compared to 76.5±2.9% in control mice (n=5-6, p=0.001). In addition, 7 days after transient ischemia, following only three days of treatment, the area of ischemic lesion was decreased by ~60% in treated mice, 15.4±1.6% *vs* 37.83±3.2%, untreated littermates, (n=6-9 p=1.8^{-4}). Note that seven days after stroke we observed some decrease in the size of the lesion in non-treated mice, which may be a result of spontaneous recovery (see Fig. 3C).

To analyze neurological deficits, the experimental animals were monitored 24- 72 hours, and than 5 and 7 days after transient MCAO. Unfortunately, 96 hr after permanent MCAO, the conditions of non-treated mice markedly deteriorated; therefore we did not analyze neurological deficits in this model beyond 72 hr. As shown in Tables 1, our treatment markedly improved clinical recovery of the mice after transient and permanent ischemia. An important additional point is that 7 days after

transient MCAO, treated mice (treatment was administered only for the first 72 hours) overall demonstrated markedly better clinical recovery than non-treated mice (See Table 1).

The three-drug therapy attenuates microglia/ macrophages and caspase-3 activation after transient but not in permanent ischemia

There is increasing evidence that post-ischemic inflammation and apoptosis contribute to ischemic brain injury and to outcome after ischemic insult (Dirnagl et al., 1998; Lo et al., 2003). To examine whether the three-drug cocktail attenuated signals for glial cell activation and apoptosis, we examined by immunohistochemistry the expression of Mac-2, a selective marker of microglial/macrophage activation, GFAP as a marker for astrogliosis and cleaved caspase-3 as a marker for apoptosis.

As demonstrated in Fig. 4, reperfusion injury induced by transient MCAO was characterized by a robust increase in microglia/macrophage immunoreactivities. The Mac-2 immunoreactive cell showed the morphology typical of activated microglia/ macrophages (irregular ameboid shape, retracted processes), localized in certain areas within the ischemic lesion, such as hippocampus and a part of the cortex, and in high numbers in the peri-infarct regions (see Fig. 4B and C). The three-drug treatment markedly attenuated Mac-2 immunoreactivity (Fig. 4E and F). Note that the microglial/macrophage activation was restricted only to the ipsilateral, ischemic side of the brain. Analysis of the GFAP immunoreactivity revealed a marked loss of GFAP immunoreactivity within the site of ischemic lesion that was associated with an increase of GFAP immuonreactivities at the peri-infarct regions (Fig 5B). As shown in the Fig 5C, our treatment attenuated the loss of GFAP immunoreactivity within the site of the lesion (see Figure 5A). Previous studies have demonstrated that neurons are particularly sensitive to caspase 3- mediated cell death. (Dirnagl, Lo).Our analysis

revealed that in brain sections of mice subjected to 60 min MCAO followed by 72 hr reperfusion period, there was a marked increase in activated caspase-3 immunoreactivity restricted to the site of the ischemic lesion (Fig. 5D and E). Treatment with the three-drug cocktail markedly attenuated cleaved caspase-3 immunoreactivity (Fig. 5F).

In contrast to the robust increase in Mac-2 immunoreactivity observed 72 hr after transient MCAO, the brain sections of mice subjected to permanent ischemia showed only a small increase, the Mac-2 immunoreactive cells being restricted to peri- infarct regions. In addition, there was no detectable difference in levels of Mac-2 immunoreactivity between the controls and the three-drug treated mice (Fig. 6A, B and C). Analysis of GFAP expression revealed a marked decrease or complete loss of GFAP immunoreactivity within the site of the ischemic lesion and the slight increase of GFAP immunoreactivity in the peri- infarct regions, in both, three-drug treated as well as untreated mice (Fig. 6 D, E and F). As further shown in Fig. 6, H and G, the brain sections from the mice subjected to permanent ischemia (72 hours after MCAO) showed only a weak increase in immunoreactivity for activated caspase-3. In addition, there was no detectable difference in the caspase- 3 immnuoreactivity in the brain sections of treated and non-treated mice. (Fig. 6, H and G).

Discussion

To date, despite successful preclinical studies, the single-agent clinical stroke trials yielded disappointing results. There are many possible explanations why clinical trials have failed. It may have been that the toxic side effects have overridden the neuroprotective potential of the compound determined in animals, limited time-window for human therapy, or simply, based on the complexity of events in cerebral ischemia, it may have been not realistic to expect that a single neuroprotective

drug will have lasting benefits. Here, we report that a treatment based on the combination of three drugs, minocycline, an antibiotic with anti-inflammatory properties, riluzole a glutamate antagonist and nimodipine voltage gated calcium channel blocker, conferred efficient neuroprotection following transient and permanent cerebral ischemia. Importantly, the drug cocktail approach conferred significantly more efficient neuroprotection than any of the cocktail components tested alone. When first administered 2 hr after transient or permanent MCAO, the three-drug cocktail significantly decreased the size of infarction (by~ 65% after transient and by ~35% after permanent ischemia). In addition, some other interesting observations emerged from this study. First, treatment with the glutamate antagonist riluzole alone was beneficial in transient, but not in the model of permanent ischemia. Second, immunohisthological analysis of the brain sections 72 hr after stroke revealed a marked difference in the inflammatory - and tissue responses to therapies between transient and permanent ischemia.

In animal models, synergistic effects have been demonstrated using neuroprotective combinations of 2 agents, including NMDA antagonists MK-801 in combinations with a GABA antagonist, caspase-3 inhibitors, free radical scavenger, growth factors and citicholine. Most of the agents listed earlier have been tested without success in the single –agent clinical trials. The question that arises is: what would be the comparative advantage of using minocycline alone or in a drug cocktail as a neuroprotective therapy in stroke? First, minocycline is a semi-synthetic tetracycline derivative that effectively crosses the blood-brain barrier and is extensively used in human with relatively few side effects (Goulden et al., 1996). Second, its capacity to alleviate several neurological disorders in animals is increasingly recognized (refs). Although the exact mechanisms of minocycline-mediated neuroprotection is still unclear, recent studies suggests that minocycline may prevent microglial

activation and reduces the induction of caspase-1, thereby decreasing the level of mature proinflammatory cytokine IL-1β, as well as caspase-3 activation (Yrjanheikki et al., 1998, 1999; Chen et al., 2000, Kriz 2002). Since inflammation and apoptosis are part of delayed tissue response to ischemic injury, the therapeutic window may be sufficiently wide for human therapy (refs). In addition, as recently suggested by Meisel et al., preventive treatment with antibacterial agent may be beneficial in stroke.

Previous studies on rats and gerbils have shown that minocycline confer neuroprotection in experimental models of cerebral ischemia (Yrjanheikki et al., 1998, 1999; Arvin et al., 2002; Wang et al., 2003). Albeit in broad agreement with previous reports of clear neuroprotection by minocycline, in our experiments, in two different experimental models, treatment with the three-drug cocktail achieved significantly better results in reducing the size of infarction than did treatment with minocycline alone, suggesting a possible synergy between different agents. Riluzole is an anti-glutamate drug whose precise mechanism of action has not been fully elucidated. It appears to interfere with the presynaptic release of glutamate, the activation of sodium channels and/or activation of G-protein coupled transduction pathways (Martin et al., 1993).In our hands, riluzole alone yielded interesting results, being beneficial in transient ischemia but ineffective in the model of permanent MCAO (see Fig.2 A and B), suggesting that glutamate release and excitotoxicity may not be a good therapeutic target in permanent ischemia (Hoyte et al., 2004). Although in our experiments treatment with nimodipine alone did not significantly decreased the size of infarction, previous findings demonstrate that L type calcium channel blockers, as add on therapy in different experimental models, were able to enhance the neuroprotective effects of glutamate antagonists MK-801 and minocyline. This may in part explain our findings that in our

previous and in the present studies the three-drug combination was more effective than any of the drugs tested alone 2002, 2003.

Another interesting observation emerged from this study. Treatment with the three-drug cocktail markedly attenuated brain inflammatory response and caspase-3 activation after transient MCAO, which may account for the neuroprotective effects in this model. However, in contrast to the strong brain tissue reaction after transient ischemia, 72 hr after permanent ischemia, we observed only a weak increase in Mac-2 and cleaved caspase-3 immunoreactivities. Intriguingly, although three-drug treatment significantly reduced the size of infarction and facilitated clinical recovery after permanent ischemia, it had no effect on the microglia/macrophage or caspase-3 activation. These findings, together with the previously described differences in the efficacy of the anti-glutamate treatment (see Fig. 2), clearly suggest that differential tissue- and therapy- responses are associated with the evolution of ischemic injury in transient and permanent ischemia, which may have important clinical implications.

In conclusion, the three-drug cocktail described here conferred efficient neuroprotection and improved clinical recovery of mice following transient and permanent ischemia. Moreover, in our experiments, the drug cocktail approach was more efficient than any of the cocktail components tested alone. Although, our treatment may confer differential neuroprotective effects in transient vs. permanent ischemia, the present study clearly suggests that simultaneous targeting of several pathophysiological pathways involved in the evolution of ischemic injury may represent a rational therapeutic approach for stroke and possibly other neurodegenerative disorders.

Acknowledgments

This research was supported by grants from the Canadian Institutes in Health Research (CIHR), Fonds de la recherche en santé Quebec (FRSQ), and FRSQ New Investigator Award (J.K.). We thank Dr. K. Krnjevic for comments.

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FIGURES

Figure 1. The histograms represent the size of the infarction expressed as a percentage of the control, the equivalent area of contralateral non-ischemic hemisphere (100%) measured 24 hours after transient, 60 min. MCAO A, or permanent MCAO, B. Minocycline 50 mg/kg, riluzole 1 mg/kg and nimodipine 0.5 mg/kg, alone or in the three-drug cocktail were adminstered 2 hours after stroke. Each value represents % of mean value ± SEM (*$p \leq 0.05$) comparison with control, ** ($p \leq 0.05$) comparison within the different treatments. (n= 6-14).

Figure 2. The histograms represent the relative size of the infarction expressed as a percentage of the control, the equivalent are of contralateral non-ischemic hemisphere (100%). Treatment with the three-drug cocktail markedly (by ~ 65%) decreased the size of infarction 72 hours after transient MCAO (60 minutes of MCAO occlusion followed by 72 hour reperfusion period) A), and by ~ 30% 72 hours of following permanent MCAO B) and by ~ 60% 7 days after transient MCAO (60 minutes of MCAO followed by 7 days of reperfusion). Each value represents mean ± SEM (*$p \leq 0.05$) (n=6-9). All treatments were initiated 2 hours after stroke and were administered for 3 days (1x day).

Figure 3. The three-drug treatment attenuates microglial/macrophages activation 72 hours following transient MCAO. Micrographs show a robust increase in Mac-2 immunoreactivity in the brain of C57Bl/6 mice subjected to 60 min MCAO followed by 72 hours reperfusion period. Increase was selective to the side of the lesion (A, B, and C). Three-drug treatment attenuated Mac-2 immunoreactivity on the ipsilateral side of the brain (D, E, F). Bar = 250 μm

Figure 4. The three-drug treatment attenuates caspase-3 activation and prevents the loss of GFAP immunoreactivity following transient MCAO. The micrographs show the immunoreactivities of GFAP in the brain sections of control (A) and at the site of ischemic lesion of the non-treated (B) and treated mice (C) 72 hours after transient MCAO. Three-drug treatment also attenuated the caspase -3 (cleaved form) immunoreactvity. Brain sections of control (D), non-treated (E) and treated (F) mice. Bar = 100 μm.

Figure 5. The three-drug treatment does not affect microglial/macrophage activation, astrogliosis and caspase-3 activation following permanent ischemia. The micrographs show the Mac-2, GFAP and cleaved caspase-3 immunorectivities in the brain sections of C57BL/6 mice 72 hours after permanent MCAO in the control mice (A, D, G), in the ischemic lesions of non-treated (B, E, H) and the three-drug treated mice (C, F, I). Bar = 100 μm.